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Journal of Pharmaceutical and Biomedical Analysis 43 (2007) 325–329
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Short communication

Development and validation of a simple HPLC method for simultaneous in vitro determination of amoxicillin and metronidazole at single wavelength

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Received 3 January 2006; received in revised form 2 June 2006; accepted 7 June 2006
Available online 21 July 2006

Abstract

A simple, rapid, sensitive and robust reversed phase-HPLC method was developed and validated to measure simultaneously the amount of amoxicillin and metronidazole at single wavelength (254 nm) in order to assess drug release profiles and drug–excipients compatibility studies for a new floating-sustained release tablet formulation and its subsequent stability studies. An isocratic elution of filtered sample was performed on C_{18} column with buffered mobile phase (pH 4.0) and UV detection at 254 nm. Quantification was achieved with reference to the external standards. The linearity for concentrations between 0.15 and 600 μg/ml for amoxicillin and 0.13 and 300 μg/ml for metronidazole were established. Intra and inter-day precision were less than 2.5%. The limits of detection (LOD) and quantification were 0.05 and 0.15 μg/ml for amoxicillin and 0.10 and 0.13 μg/ml for metronidazole. The determination of the two active ingredients was not interfered by the excipients of the products. Samples were stable in the release media (37 °C) and the HPLC injector at least for 12 h.
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Keywords: HPLC; Amoxicillin; Metronidazole; Simultaneous determination; Sustained release; Method validation

1. Introduction

At the current time, a combination of amoxicillin, a broad-spectrum amino-substituted β-lactam penicillin and metronidazole, a synthetic 5-nitroimidazole is used commonly with other drugs, for the eradication of *Helicobacter pylori*, which is accepted as the most important cause of gastritis and peptic ulcer disease [1–3]. For development of new drug delivery systems containing these antibiotics and comparing of different formulations consisting of these active ingredients, it is desirable to quantify their aqueous concentrations simultaneously during in vitro studies. Several HPLC methods have been reported for the individual assay of these two antibiotics in various media [4–10]. A method for measurement of these two drugs in a single plasma sample has been reported but it requires a different UV wavelength for each analyte (230 nm for amoxicillin and 313 nm for metronidazole) [11]. The novelty of this report is that simultaneous determination of these two antibiotics for in vitro analysis at single wavelength (254 nm) has not been reported, yet.

RP-HPLC assay of metronidazole is relatively straightforward, while for amoxicillin there are particular difficulties mainly due to its amphoteric nature causing it to elute among other polar substances. Furthermore, high polarity of drug makes it unstable in media of high or low pH [12].

This article introduces a simple, specific, precise and robust HPLC method which has been developed and validated based on FDA guidance and the ICH guidelines Q2A and Q2B [13–17] to measure simultaneously the amount of both drugs in order to assess their release profiles from new floating-sustained release tablet formulations.

2. Experimental

2.1. Chemicals

All reagents and solvents were of analytical and HPLC grade and included hydrochloric acid, monobasic potassium phosphate

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doi:10.1016/j.jpba.2006.06.002

(KH_2PO_4), sodium hydroxide and methanol (all from Merck, Darmstadt, Germany). Amoxicillin (as trihydrate) (from DSM Deretil, Spain) and metronidazole (from Jinxin Pharmaceutical, China) of the highest grade (purity > 99.0%) which have been standardized with relative USP Reference Standards and considered as external standards. All the excipients used for the development of placebo formulations were obtained from commercial sources and were used as such. Double distilled water was used during the entire HPLC procedure.

2.2. Instrumentation

The HPLC system consisted of a separation module (Alliance 2690) and photodiode array (PDA) detector (2487), all from Waters (Waters Corporations, USA). An isocratic elution was performed on a Whatman® Partisil 5 ODS-3 column (100 mm × 4.6 mm) (from Whatman International Ltd., England), 5 μm particle size, plus Whatman® guard cartridge (RP cartridge). The mobile phase was a degassed and filtered (0.45 μm; Millipore) mixture of phosphate buffer solution (pH 4.7; 0.05 M)–methanol (95:5, v/v) with final pH adjusted to approximately 4.0.

Injection volumes were 10 μl and flow-rate was established on 1.5 ml/min. The UV detector wavelength was set at 254 nm for the determination of both amoxicillin and metronidazole. Integration of the chromatography response was carried out using Millenium32® software. Quantification was achieved by the peak-area ratio method with reference to the external standard.

2.3. Sample preparation

Stock standard solutions of amoxicillin and metronidazole were freshly prepared in monobasic potassium phosphate buffer (pH 4.7; 0.05 M) at concentrations of 600 and 300 μg/ml, respectively, as external standards. For linearity study, nine level of working standard dilutions (including limits of quantitation, LOQ) were prepared from mixture of amoxicillin/metronidazole in monobasic potassium phosphate buffer (pH 4.7; 0.05 M) at concentrations ranging from 0.15 to 600 μg/ml (for amoxicillin) and 0.13 to 300 μg/ml (for metronidazole). For the recovery procedure, quality control (QC) samples were made by addition of the determined quantity of stock solution to drug-free (placebo) formulation.

2.4. Validation criteria

2.4.1. Specificity

The specificity defined as the ability of method to measure the analyte accurately and specifically in the presence of components present in the sample matrix, was determined by analysis of chromatograms of drug-free and drug-added placebo formulation.

2.4.2. Linearity

Nine-points standard curves for both compounds were constructed by drawing peak area versus amoxicillin or metronidazole concentration using 0.15–600 μg/ml (for amoxicillin) and 0.13–300 μg/ml (for metronidazole) processed separately and run in duplicate daily on the 3 consecutive days. The concentration ranges were selected based on drug concentration levels during the release studies.

Calibration curves were generated using weighted linear regression analysis with a weighting factor of $1/x$ over the respective standard concentration range.

2.4.3. Accuracy and precision

The accuracy of an analytical method is defined as the similarity of the results obtained by the analytical method to the true value and the precision as the degree of that similarity [18]. Accuracy of the method was performed by recovery study of three drug-spiked placebo formulations of five concentrations (one near to the limits of quantitation) on a single assay day to determine intra-day precision and accuracy. In addition, analyses of five samples of five concentrations on 3 consecutive days were used to determine inter-day precision and accuracy.

Recovery studies of amoxicillin and metronidazole were performed using the method of standard addition for measuring accuracy of method. Five concentrations in the range of 300–0.15 μg/ml of amoxicillin and 150–0.13 μg/ml of metronidazole were made by adding sufficient amount of standard solution to suspensions containing constant amount of ingredients of tablet formulations, had been developed for floating tablets. The prepared suspensions were filtered and then injected to the HPLC system.

The assessment of assay precision was carried out using the data from the recovery study. The method of analysis of variance (ANOVA) was used for estimating the total variability, and between and within day variability of the analytical method.

The reference solution was analyzed 10 times and relative standard deviation was determined via the peak area as measuring precision.

2.4.4. Limits of detection (LOD) and quantitation (LOQ)

The detection limit (LOD) and quantification limit for each analyte were determined based on signal-to-noise concept, as the lowest concentrations at which signal-to-noise ratio is between 3 or 2:1 and 10:1, respectively, with defined precision and accuracy under the given experimental conditions.

2.5. Stability

Drug stability should be considered in analysis of in vitro extended release data, since released drug may degrade in due time [19].

The stability of amoxicillin and metronidazole was determined both in the injector and in the release medium. Replicate standards at concentrations of 600 and 300 μg/ml, respectively, were injected at evenly spaced intervals during a single HPLC assay run of 12 h and secondly, the stability of both analytes was assessed at the same concentrations in the release medium of monobasic potassium phosphate buffer (pH 4.7; 0.05 M, 37 °C) at 3 h intervals until 12 h.

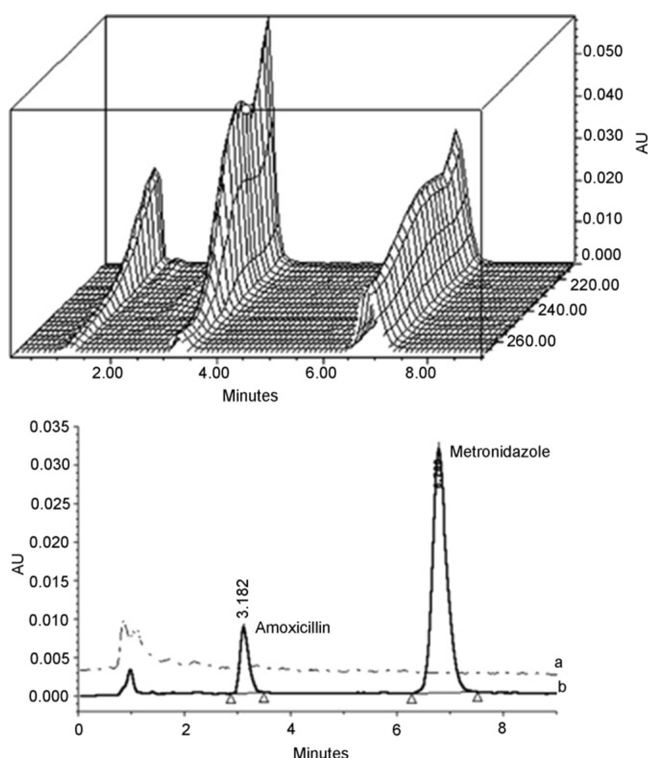

Fig. 1. 3D (upper) and 2D (lower) representative chromatograms of a blank QC sample (a) and of simultaneously analyzed two drugs-spiked samples (b), amoxicillin (peak at 3.2 min) and metronidazole (peak at 6.8 min) at 254 nm.

3. Results and discussion

3.1. Specificity

Fig. 1 shows a three and two-dimensional chromatogram for both drugs indicating no interference between two drugs at 254 nm. Good separation is seen as the retention times of amoxicillin and metronidazole were around 3.2 and 6.8 min, respectively, using the chromatographic conditions described above. No interfering peaks were encountered in the blank samples.

Moreover, representative chromatograms of a blank placebo sample (formulation without drug) (a) and of two drugs-spiked samples (b) are depicted. No interference between two drugs and excipients is depicted at this wavelength. The total chromatography run time was prolonged by 10 min to allow for a late eluting peak. The chromatographic run time of 10 min was sufficient for sample analysis that allows analyzing large number of samples in a short period of time. Injection of blank phosphate buffer into HPLC column represented that no peak could be seen on chromatogram.

3.2. Linearity and calibration

Based on 1/x weighted linear regression analysis, the responses for both drugs in related concentration ranges were linear and calibration equations were $y = 1013.78$ ($\pm$25.12)$x + 700.82$ ($\pm$46.08) ($n = 54$, $R^2 = 0.9992$) for amoxicillin and $y = 4835.57$ ($\pm$129.95)$x - 372.22$ ($\pm$20.66) ($n = 54$, $R^2 = 0.9992$) for metronidazole.

The R.S.D.s of the slopes were $\leq$2.7% for both analytes ($n = 54$).

Table 1 shows the back-fit calculations for standard curve data used in validation runs, as well as the precision and accuracy of the back-fit calculations.

3.3. Assay precision and accuracy

The mean observed concentrations for all QC samples were calculated based on calibration curves. The repeatability (intra-assay precision) and the intermediate (between-assay or inter-assay) precision were calculated from data obtained during 3 days validation. Results are shown in Tables 2 and 3.

The recovery of these two active ingredients from the spiked samples (including LOQ sample) was at the range of 92.31–102.93% and lies within the recommended tolerance of 80–115% [20].

The intra-day precisions of three replicates on the same day for four concentrations were $\leq$1.3 and for LOQ concentrations were $\leq$2.5. The inter-day precisions for five replicates in 3 consecutive days were $\leq$0.8% for both analytes at four concentrations and for LOQ were $\leq$2.5. From Tables 2 and 3, it appears that both precision and accuracy were within acceptable

Table 1
Curve parameter summary and back-calculated calibration curve concentrations for amoxicillin and metronidazole

	Amoxicillin concentration (μg/ml)								
	0.15	**0.30**	**1.5**	**6**	**15**	**60**	**150**	**300**	**600**
Mean ($n = 6$)	0.14	0.31	1.57	5.57	14.84	62.08	155.46	311.67	616.62
S.D.±	0.00	0.01	0.02	0.04	0.07	0.02	0.32	0.14	1.93
R.S.D. (%)	2.60	2.26	1.01	0.73	0.47	0.04	0.20	0.04	0.31
Bias (%)	−7.67	3.33	4.83	−7.15	−1.06	3.46	3.64	3.89	2.77
	Metronidazole concentration (μg/ml)								
	0.13	**0.150**	**0.75**	**3**	**7.5**	**30**	**75**	**150**	**300**
Mean ($n = 6$)	0.12	0.15	0.75	3.08	7.17	29.47	73.66	148.90	298.02
S.D.±	0.00	0.00	0.00	0.01	0.12	0.13	0.28	0.21	0.83
R.S.D. (%)	2.02	1.59	0.64	0.42	1.69	0.45	0.38	0.14	0.28
Bias (%)	−4.31	1.73	0.10	2.55	−4.40	−1.75	−1.78	−0.74	−0.66

Table 2
Intra-assay variability of amoxicillin and metronidazole obtained from five levels of QC samples

Concentration added (μg/ml)	Concentration found (mean ± S.D., $n = 3$) (μg/ml)	Precision (%R.S.D.)	Accuracy (%)
Amoxicillin			
0.15	0.14 ± 0.00	2.5	93.06
15.0	15.08 ± 0.2	1.3	100.53
60.0	61.53 ± 0.21	0.4	102.55
150.0	154.20 ± 1.69	1.1	102.79
300.0	305.55 ± 1.35	0.4	101.85
Metronidazole			
0.13	0.12 ± 0.00	2.3	93.30
7.50	7.72 ± 0.04	0.5	102.93
30.0	30.52 ± 0.19	0.6	101.73
75.0	75.63 ± 0.93	1.2	100.84
150	151.83 ± 0.82	0.5	101.22

limits for routine drug analysis (15%). As repeatability, precision data for six replicates at concentrations of 60 and 600 μg/ml of amoxicillin were ≤1.5% and at 30 and 300 μg/ml of metronidazole were ≤2.0%, which lie within recommended tolerance of ≤2.0%. The measuring precisions were determined ≤0.7 and ≤0.2% for amoxicillin and metronidazole, respectively, after six injections as an indicator of instrument precision lies within recommended tolerance of ≤1.0%.

Shah et al. [21] recommended that an analytical method may be considered validated in terms of accuracy if the mean value is within ±15% of the actual value. Similarly, it is also suggested that the analytical method may be considered validated in terms of precision if the precision around the mean value dose not exceed a 15% coefficient of variation (CV or R.S.D.) [21].

3.4. Limits of detection and quantitation

The limits of detection and quantification decide about the sensitivity of the method and were calculated from the peak-to-noise ratios. In the present study, the LOQ values for amoxicillin and metronidazole were 0.15 and 0.13 μg/ml and detection limits were found to be 0.05 and 0.10 μg/ml, respectively.

Table 3
Inter-assay variability of amoxicillin and metronidazole obtained from five levels of QC samples

Concentration added (μg/ml)	Concentration found (mean ± S.D., $n = 5$) (μg/ml)	Precision (%R.S.D.)	Accuracy (%)
Amoxicillin			
0.15	0.14 ± 0.00	2.5	92.40
15	14.90 ± 0.06	0.4	99.33
60	59.88 ± 0.02	0.0	99.80
150	151.88 ± 1.26	0.8	101.25
300	299.09 ± 0.39	0.1	99.69
Metronidazole			
0.13	0.12 ± 0.00	2.3	92.31
7.5	7.41 ± 0.01	0.1	98.80
30	30.29 ± 0.13	0.4	101.0
75	74.90 ± 0.58	0.8	99.87
150	149.66 ± 0.20	0.1	99.8

3.5. Stability

In the case of an unexpected delay during analysis, it is important to have information about the stability of all solutions. In this study, the stability of amoxicillin in the working standard solution and sample preparations were studied. The responses for the aged solutions were evaluated using a freshly prepared standard. Amoxicillin and metronidazole did not show evidence of significant degradation for at least 12 h, when kept at release medium (monobasic potassium phosphate buffer solution, pH 4.7; 0.05 M, 37 °C) and in auto-sampler (10 °C). During this period, results do not decrease below 95%. The lack of instability allowed a large batch of samples to be processed in one assay run.

4. Conclusion

An HPLC method for simultaneous determination of amoxicillin and metronidazole for in vitro analysis at single wavelength has not been reported. The presented method in addition to its novelty for determination of two ingredients at single wavelength is sufficiently rapid, simple and sensitive as well as precise and accurate that complies with FDA guidelines for accuracy, precision and stability for standards and QC samples. The assay of the two active ingredients was not interfered by the excipients in the products. The linearity, accuracy, precision, limit of detection and quantification, specificity–selectivity of the method and sample stability were established. In addition to analysis of drug during assay, dissolution and release studies, this rapid and reproducible analytical method was successfully used for analysis of drug–excipient compatibility study of a new sustained release formulations prepared in our laboratory and their subsequent stability studies.

Acknowledgement

The authors express sincere thanks to FARABI Pharmaceutical Co. Isfahan, Iran for their cooperation and supply of some consumables.

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